Comparative Transcriptome Analysis Reveals the Mechanism Underlying 3,5-Dibromo-4-Hydroxybenzoate Catabolism via a New Oxidative Decarboxylation Pathway

Kai Chen,^{a,c} Yang Mu,^a Shanshan Jian,^a Xiaoxia Zang,^a Qing Chen,^a Weibin Jia,^a Zhuang Ke,^a Yanzheng Gao,^c Jiandong Jiang^{a,b}

^aDepartment of Microbiology, Key Lab of Environmental Microbiology for Agriculture, Ministry of Agriculture, College of Life Sciences, Nanjing Agricultural University, Nanjing, China

^bJiangsu Key Lab for Solid Organic Waste Utilization, Nanjing, China

^cInstitute of Organic Contaminant Control and Soil Remediation, College of Resource and Environmental Sciences, Nanjing Agricultural University, Nanjing, China

ABSTRACT The compound 3,5-dibromo-4-hydroxybenzoate (DBHB) is both anthropogenically released into and naturally produced in the environment, and its environmental fate is of great concern. Aerobic and anaerobic reductive dehalogenations are the only two reported pathways for DBHB catabolism. In this study, a new oxidative decarboxylation pathway for DBHB catabolism was identified in a DBHB-utilizing strain, *Pigmentiphaga* sp. strain H8. The genetic determinants underlying this pathway were elucidated based on comparative transcriptome analysis and subsequent experimental validation. A gene cluster comprising *orf420* to *orf426*, with transcripts that were about 33- to 4,400-fold upregulated in DBHB-induced cells compared with those in uninduced cells, was suspected to be involved in DBHB catabolism. The gene *odcA* (*orf420*), which is essential for the initial catabolism of DBHB, encodes a novel NAD(P)H-dependent flavin monooxygenase that mediates the oxidative decarboxylation of DBHB to 2,6-dibromohydroquinone (2,6-DBHQ). The substrate specificity of the purified OdcA indicated that the 4-hydroxyl group and its *ortho*-halogen(s) are important for hydroxylation of the C-1 site carboxyl group by OdcA. 2,6-DBHQ is then ring cleaved by the dioxygenase OdcB (Orf425) to 2-bromomaleylacetate, which is finally transformed to β -ketoadipate by the maleylacetate reductase OdcC (Orf426). These results provide a better understanding of the molecular mechanism underlying the catabolic diversity of halogenated *para*-hydroxybenzoates.

IMPORTANCE Halogenated hydroxybenzoates (HBs), which are widely used synthetic precursors for chemical products and common metabolic intermediates from halogenated aromatics, exert considerable adverse effects on human health and ecological security. Microbial catabolism plays key roles in the dissipation of halogenated HBs in the environment. In this study, the discovery of a new catabolic pathway for 3,5-dibromo-4-hydroxybenzoate (DBHB) and clarification of the genetic determinants underlying the pathway broaden our knowledge of the catabolic diversity of halogenated HBs in microorganisms. Furthermore, the NAD(P)H-dependent flavin monooxygenase OdcA identified in *Pigmentiphaga* sp. strain H8 represents a novel 1-monooxygenase for halogenated *para*-HBs found in prokaryotes and enhances our knowledge of the decarboxylative hydroxylation of (halogenated) *para*-HBs.

KEYWORDS halogenated *para*-hydroxybenzoate, decarboxylative hydroxylation, *Pigmentiphaga* sp., flavin monooxygenase, comparative transcriptome

Received 6 November 2017 Accepted 13 December 2017

Accepted manuscript posted online 5 January 2018

Citation Chen K, Mu Y, Jian S, Zang X, Chen Q, Jia W, Ke Z, Gao Y, Jiang J. 2018. Comparative transcriptome analysis reveals the mechanism underlying 3,5-dibromo-4-hydroxybenzoate catabolism via a new oxidative decarboxylation pathway. *Appl Environ Microbiol* 84:e02467-17. <https://doi.org/10.1128/AEM.02467-17>.

Editor Haruyuki Atomi, Kyoto University

Copyright © 2018 American Society for Microbiology. All Rights Reserved.

Address correspondence to Jiandong Jiang, jiang_jjd@njau.edu.cn.

Halogenated hydroxybenzoates are widely used as synthetic precursors for industrial and agricultural chemicals and are common metabolic intermediates from halogenated aromatics, such as polychlorobiphenyls and halogenated polycyclic aromatic hydrocarbons. Because of their toxicity, persistence, and bioaccumulation, the presence of halogenated hydroxybenzoates in the environment exerts adverse effects on human health and ecological security (1–3). The compound 3,5-dibromo-4-hydroxybenzoate (DBHB), a metabolic intermediate of the commonly used herbicide bromoxynil, is frequently detected in soil, groundwater, animal and plant tissues, and even milk (4–8). In addition to the anthropogenic release of DBHB into the terrestrial environment, DBHB is also naturally biosynthesized in the marine environment by many marine organisms, such as the green alga *Ulva lactuca*, the red alga *Symphyocladia latiuscula*, and the sponge *Psammaplysilla purpurea* (9–11). Although DBHB is reported to have less toxicity than its parent compound bromoxynil, DBHB can still cause liver and developmental effects and inhibit seed germination and the growth of soil microorganisms (12).

Due to its anthropogenic release and natural production, the environmental fate of DBHB has attracted considerable attention (6, 11, 13). Microorganisms play key roles in the dissipation of DBHB in the environment, and many bacterial strains are capable of catabolizing DBHB (13–15). Two DBHB catabolic pathways have been identified in bacteria (Fig. 1): (i) the anaerobic reductive dehalogenation pathway, in which DBHB is successively dehalogenated to the end product *para*-hydroxybenzoate (4-HB) via an unknown reductive dehalogenase in the anaerobic strain of *Desulfitobacterium chlororespirans* (14), and (ii) the aerobic reductive dehalogenation plus oxidative ring cleavage pathway, in which DBHB is successively dehalogenated to 4-HB by a nonrespiratory reductive dehalogenase (BhbAB or its homologs), and 4-HB is transformed to 4-carboxy-2-hydroxymuconate-6-semialdehyde by 4-hydroxybenzoate 3-monooxygenase (BhbF2) and protocatechuate 4,5-dioxygenase (BhbD2E2) in the aerobic strains *Comamonas* sp. strain 7D-2 and *Delftia* sp. strain EOB-17 (13, 15, 16). It needs to be mentioned that 3,5-dichloro-4-hydroxybenzoate, the chlorine-substituted counterpart of DBHB, has been reported to be transformed to 2,6-dichlorohydroquinone in *Pseudomonas* sp. strain HH35 (17). However, the genetic basis for this oxidative decarboxylation process remains unknown. In general, the catabolic diversity of DBHB and its genetic background are far from clear.

In this study, the oxidative decarboxylation pathway for DBHB catabolism was identified in a DBHB-utilizing strain *Pigmentiphaga* sp. strain H8, and the molecular mechanism of this catabolic pathway was clarified via comparative transcriptome analysis (RNA-seq) and biological experimental validation. A gene cluster consisting of *orf420* to *orf426* was found to be over 30-fold upregulated in response to DBHB induction and to be involved in DBHB catabolism. The emphasis of this study was on the investigation of a novel 1-monooxygenase (OdcA) which catalyzes the initial oxidative decarboxylation of DBHB to 2,6-dibromohydroquinone (2,6-DBHQ).

RESULTS

Degradation of DBHB by *Pigmentiphaga* sp. strain H8. Strain H8 (uninduced) degraded 0.20 mM DBHB to undetectable levels in minimal salts medium (MSM) within 24 h, and the initial culture density (optical density at 600 nm [OD₆₀₀]) increased from 0.08 to 0.12, showing that strain H8 was capable of utilizing DBHB as a sole source of carbon and energy for growth (Fig. 2A). Additionally, 0.37 mM bromide was released during the degradation of 0.20 mM DBHB, suggesting that both bromines were cleaved from DBHB by strain H8.

The DBHB-induced cells of strain H8 (OD₆₀₀ = 0.3) completely degraded 0.20 mM DBHB within 6 h (Fig. 2B). Uninduced cells degraded less than 12.5% of DBHB during the first 6 h and degraded approximately 75% of DBHB at 12 h. Thus, the degradation of DBHB by strain H8 was induced by DBHB, suggesting the possibility of identifying DBHB catabolic genes using comparative transcriptome analysis.

FIG 1 Previously reported and newly proposed DBHB catabolic pathways. (A) Anaerobic reductive dehalogenation pathway in *Desulfotobacterium chlororespirans*. (B) Aerobic reductive dehalogenation plus oxidative ring cleavage pathway in *Comamonas* sp. strain 7D-2 and *Delftia* sp. strain EOB-17. (C) Oxidative decarboxylation pathway in *Pigmentiphaga* sp. strain H8, which is newly proposed in this study. (D) Gene cluster consisting of *orf420* to *orf426*, which is involved in DBHB catabolism. The arrows indicate the size and direction of transcription of each *orf*. *orf420* (*odcA*), DBHB 1-monooxygenase gene; *orf421*, putative muconic semialdehyde dehydrogenase gene; *orf422*, putative regulator gene; *orf423*, putative transporter gene; *orf424*, hypothetical protein gene; *orf425* (*odcB*), 2,6-dibromohydroquinone 1,2-dioxygenase gene; *orf426* (*odcC*), putative maleylacetate reductase gene. Abbreviations: DBHB, 3,5-dibromo-4-hydroxybenzoate; 2,6-DBHQ, 2,6-dibromohydroquinone; DBHODA, 2,6-dibromo-4-hydroxy-6-oxohexa-2,4-dienoic acid; TCA, tricarboxylic acid.

Prediction of DBHB catabolic genes based on comparative transcriptome analysis.

Comparative transcriptome analysis revealed that 98 transcripts (~1.7% of the total transcripts in strain H8) were over 10-fold upregulated and 31 transcripts were over 30-fold upregulated in DBHB-induced cells compared with those in uninduced cells (see Table S1 in the supplemental material). Based on the annotations of 31 genes using BLASTP, a gene cluster consisting of *orf420* to *orf426* (about 33 to 4,400-fold upregulated) was suspected to be involved in DBHB catabolism (Table 1). In this gene cluster, *orf420* (named *odcA*) is speculated to encode a flavin monooxygenase which shows the highest identities (at the amino acid sequence level) to PnpA (36%) and HspB (34%), catalyzing the hydroxylation of a nitro or succinoyl group of *para*-nitrophenol and 6-hydroxy-3-succinoylpyridine, respectively (18, 19), *orf425* (*odcB*) encodes a dioxygenase with 62% identity to 2,6-dichloro-*p*-hydroquinone 1,2-dioxygenase (PcpA) (20, 21) and 49% identity to chlorohydroquinone/hydroquinone 1,2-dioxygenase (LinE) (22), and *orf426* (*odcC*) encodes a reductase with 54% identity to maleylacetate reductases

FIG 2 Degradation of DBHB in MSM by *Pigmentiphaga* sp. strain H8 (A), DBHB-induced and uninduced strain H8 (B), and mutant H8ΔodcA and complementary strain H8ΔodcA (pMCS-odcA) (C) under aerobic conditions. The initial OD₆₀₀ values of the inoculated cells for panels A, B, and C were 0.08, 0.3, and 0.5, respectively. The cell growth of strain H8 and bromide ion release were also measured for panel A. Data are represented as the mean $\pm$ standard deviation for triplicate (A and C) or quadruplicate (B) experiments. When the error bar is not visible, it is within the data point.

TfE and MacA (23, 24). In addition, the protein encoded by *orf422* shows 36% identity to the HTH-type transcriptional activator NahR (25). To confirm whether these predicted genes are responsible for DBHB catabolism, the functions of Orf420 (OdcA), Orf425 (OdcB), and Orf426 (OdcC) were experimentally validated.

***orf420 (odcA)* is essential for initial degradation of DBHB in strain H8.** The mutant H8ΔodcA (with a *odcA* gene knockout) completely lost its ability to degrade DBHB, and *odcA* gene complementation of the mutant H8ΔodcA (transformed with the plasmid pMCS-odcA) recovered the ability to catabolize DBHB (Fig. 2C), showing that the *odcA* gene is essential for the initial degradation of DBHB.

TABLE 1 Gene cluster suspected to be involved in DBHB catabolism with transcripts with over 30-fold upregulation in DBHB-induced cells compared with those in uninduced cells

orf (gene)	Product size ^a	Fold change ^b	Database ^c	Protein with highest identity, function, host	GenBank accession no.	Identity (%)
<i>orf420 (odcA)</i>	402	565.8	Swiss-Prot	PnpA, conversion of <i>para</i> -nitrophenol to benzoquinone in the presence of NADPH, <i>Pseudomonas</i> sp. strain WBC-3	C11201	36
			Swiss-Prot	HspB, conversion of 6-hydroxy-3-succinylpyridine (a metabolite of nicotine) to 2,5-dihydroxypyridine in the presence of NADH, <i>P. putida</i> S16	F8G0M4	34
<i>orf421</i>	488	680.1	Swiss-Prot	2-Hydroxymuconic semialdehyde dehydrogenase (DmpC), conversion of 2-hydroxymuconic semialdehyde to 2-hydroxymuconic acid, <i>Pseudomonas</i> sp. strain CF600	P19059	55
<i>orf422</i>	299	32.7	Swiss-Prot	HTH-type transcriptional activator (NahR), activator of transcription of naphthalene and salicylate catabolic gene clusters, <i>P. putida</i>	P10183	36
<i>orf423</i>	326	444.6	Swiss-Prot	UPF0065 protein in <i>tcbD-tcbE</i> intergenic region, <i>Pseudomonas</i> sp. strain P51	P27103	38
<i>orf424</i>	262	1,787.9	Swiss-Prot	Putative aminoacylate hydrolase (RutD), <i>Methylobacterium extorquens</i> CM4	B7KWT4	36
<i>orf425 (odcB)</i>	320	4,399.5	PDB	PcpA, conversion of 2,6-dichloro- <i>p</i> -hydroquinone to 2-chloromaleylacetate, <i>Sphingobium chlorophenolicum</i> L-1	AAC64295	62
			Swiss-Prot	LinE, conversion of 2-chlorohydroquinone to maleylacetate, <i>Sphingomonas paucimobilis</i> UT26	Q9WXE6	49
<i>orf426 (odcC)</i>	365	371.8	Swiss-Prot	TfE, catalysis of reduction of 3-chloro-maleylacetate to 3-chloro- β -ketoacid, <i>Burkholderia cepacia</i> AC1100	Q45072	54
			NR	MacA, catalysis of NADH-dependent reduction of 2-chloromaleylacetate to β -ketoacid, <i>Burkholderia cepacia</i> AC1100	AAD55886	54

^aNumber of amino acids.

^bAverage ratio of expression in DBHB-induced cells to that in DBHB-uninduced cells from two biological replicates for RNA-seq (mRNA); FPKM values with fold changes of ≥ 30 (DBHB induced/uninduced) are considered significantly more abundant in the presence of DBHB ($P < 0.05$).

^cNR, NCBI nonredundant protein sequence database; PDB, Protein Data Bank proteins.

FIG 3 Phylogenetic analysis of *Odca* reveals its relationship to other flavin monooxygenases from groups A to H. GenBank accession numbers or protein IDs are shown in parentheses after each protein. The bar represents 0.2 amino acid substitution per site.

***Odca* is a novel flavin monooxygenase capable of oxidative decarboxylation of DBHB to 2,6-DBHQ.** Phylogenetic analysis showed that *Odca* is closely related to *PnpA* and *HspB* but forms a separate clade from the functionally similar *para*-hydroxybenzoate 1-monooxygenases/hydroxylases (*MNX1*, [G8B709](#); and *VibMO1*, [ANB32222](#)) and *ortho*-hydroxybenzoate 1-monooxygenases/hydroxylases (*Sal*, [Q59713](#); *SalA*, [Q9RBI2](#); *NahG*, [P23262](#); and *NahW*, [Q9Z164](#)), although these proteins all clustered within the group A flavin monooxygenases (Fig. 3). Sequence analysis showed that *Odca* contains a Rossmann motif, GxGxxG(x)₁₇E, for flavin adenine dinucleotide (FAD) binding and uses NAD(P)H as the electron donor, which is a feature of the group A flavin monooxygenases (26, 27).

No signal peptide was found in the N-terminal region of *Odca*. *Odca* was overexpressed in *Escherichia coli* BL21(DE3) as an N-terminal Strep II (WSHPQFEK)-tagged fusion protein. Approximately 560 μ g of soluble and active Strep-*Odca* was purified from 0.73 g of cells (wet weight) via Strep-Tactin affinity chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed that the molecular mass of the denatured Strep-*Odca* was approximately 46 kDa (including the Strep tag) (see Fig. S1 in the supplemental material), which agreed with the deduced molecular mass from the amino acid sequence (45,664 Da).

To confirm the function of *Odca* against DBHB, the consumption of both DBHB and NADPH in an enzymatic reaction system was measured by performing UV spectrum

FIG 4 UV spectrum scanning of DBHB transformation by purified OdcA (A) and of 2,6-DBHQ transformation by *E. coli* DH5 α (pUC-odcB) (B). The spectra in panel A were taken at reaction times of 0, 1, 2, 3, 4, 5, 6, 7, 8, and 9 min, and the spectra in panel B were taken at 0, 1, 2, 3, 4, 5, 6, and 7 h. The arrows show the directions of spectral changes.

scanning. Rapid degradation of DBHB (λ_{max} , 221 nm) by purified Strep-OdcA with continuous consumption of NADPH (λ_{max} , 340 nm) was observed (Fig. 4A). In addition, an isosbestic point at 250 nm increased, indicating that a new metabolite was produced during DBHB degradation. Furthermore, no significant degradation of DBHB by OdcA under anaerobic conditions was observed, showing that oxygen is necessary for the activity of the OdcA (see Fig. S2 in the supplemental material).

The metabolite produced from DBHB degradation by purified Strep-OdcA was further identified by performing gas chromatography (GC)-mass spectrometry (MS). The metabolite was first acetylated and showed a retention time of 17.4 min in the GC trace (Fig. 5A), which matched 2,6-DBHQ in the NIST library with 74% probability. The metabolite, which showed a molecular ion peak at m/z 352.0 and fragments at m/z 309.9 (loss of —COCH_3) and m/z 268.0 (loss of two —COCH_3) by MS (Fig. 5A, inset), was identified as acetylated 2,6-DBHQ. Peaks at m/z 266.0 [$M - 2$], 268.0 [M], and 269.9 [$M + 2$] and a relative intensity ratio of 1:2:1 are characteristic of a molecule with two bromine atoms. Based on the above results, OdcA was confirmed to be a monooxygenase capable of converting DBHB to 2,6-DBHQ via oxidative decarboxylation.

Characteristics of OdcA. No activity was detected for purified Strep-OdcA in the absence of any cofactors (Table 2), but the addition of NADPH or NADH greatly increased OdcA activity. When NADPH was used as a cofactor, the relative activity of OdcA was approximately 3-fold higher than that of NADH. Supplementation of FAD with NAD(P)H further promoted OdcA activity. These results indicate that OdcA is a FAD-binding protein and utilizes NAD(P)H as an electron donor in the oxidative decarboxylation process. The optimum temperature for the activity of OdcA was 35°C, and the optimum pH was at 7 (see Fig. S3 in the supplemental material).

Substrate range of OdcA. Enzyme kinetic tests showed that OdcA had a K_m of $32.4 \pm 2.9 \mu\text{M}$ and a K_{cat}/K_m ratio of $4.2 \pm 0.2 \mu\text{M}^{-1} \text{min}^{-1}$ for DBHB, as well as a K_m of $187.9 \pm 13.1 \mu\text{M}$ and a K_{cat}/K_m ratio of $0.09 \pm 0.01 \mu\text{M}^{-1} \text{min}^{-1}$ for 3-bromo-4-hydroxybenzoate (BHB). In addition to DBHB and BHB, the chlorinated 4-hydroxybenzoate counterparts 3,5-dichloro-4-hydroxybenzoate (DCHB) and 3-chloro-4-hydroxybenzoate (CHB) were also decarboxylated by OdcA, as summarized in Table 3. 3,5-Dibromobenzoate was not a substrate of OdcA, showing that the 4-hydroxyl group was necessary for the hydroxylation of the C-1 site carboxyl group. Interestingly, 4-hydroxybenzoate was not transformed by OdcA at any detectable level, indicating that the halogen at the *ortho* position to the 4-hydroxyl group

FIG 5 Identification of the metabolites produced during the catabolism of DBHB. (A) GC-MS chromatogram analysis of the acetylated metabolite produced from DBHB by purified Strep-OdcA. The mass spectrum of the GC peak at 17.4 min was identical to that of acetylated 2,6-DBHQ. (B) LC-MS chromatogram analysis of the metabolite produced from 2,6-DBHQ by *E. coli* DH5 α (pUC-odcB). The main peak at 2.2 min gives the spectrum, which was identical to that of 2-bromomaleylacetate. (C) GC-MS chromatogram analysis of the trimethylsilylated end metabolite produced from DBHB by *E. coli* DH5 α (pUC-odcABC). The mass spectrum of the GC peak at 18.1 min was identical to that of trimethylsilylated β -ketoadipate.

TABLE 2 Relative activities of OdcA with different cofactors

Cofactor(s) with OdcA	Relative activity (%) ^a
None	0
NADPH	100.0 ± 4.0
NADPH + FAD	162.9 ± 1.1
NADPH + FMN	87.8 ± 1.6
NADH	33.5 ± 1.9
NADH + FAD	103.1 ± 3.0
NADH + FMN	27.3 ± 0.7
FAD	0
FMN	0

^aThe relative activity of OdcA with 0.3 mM NADPH as the cofactor was set at 100%. Values are means ± standard deviations.

was also important. Only the carboxyl group was hydroxylated; the nitro group (e.g., 4-nitrophenol, 2-chloro-4-nitrophenol, and 2,6-dichloro-4-nitrophenol), nitrile group (3,5-dibromo-4-hydroxybenzonitrile), chlorine group (2,4,6-trichlorophenol), and amine group (3,5-dichloro-4-hydroxyaniline) were not hydroxylated by OdcA. In addition, 6-hydroxy-3-succinoylpyridine, the substrate of HspB, was not transformed by OdcA either.

Ofr425 (OdcB) catalyzes the ring cleavage of 2,6-DBHQ to form 2-bromomaleylacetate. To determine whether 2,6-DBHQ was ring cleaved by Ofr425 (OdcB), 2,6-DBHQ was transformed by *E. coli* DH5α(pUC-odcB). A decrease in 2,6-DBHQ (λ_{max} , 298 nm) and increase in product (λ_{max} , 253 nm) were observed by UV spectrum scanning (Fig. 4B), while no such change was observed for the negative control (*E. coli* DH5α harboring pUC18). The continuous change in the UV spectrum of 2,6-DBHQ catalyzed by OdcB agreed with that of 2,6-dichloro-*p*-hydroquinone catalyzed by PcpA to produce 2-chloromaleylacetate (20). In addition, no significant degradation of 2,6-DBHQ by *E. coli* DH5α(pUC-odcB) in the absence of oxygen was observed, indicating that OdcB is an oxygenase (Fig. S2).

The metabolite produced from 2,6-DBHQ by *E. coli* DH5α(pUC-odcB) was further identified by liquid chromatography-mass spectrometry (LC-MS). The metabolite (retention time [RT] = 2.2 min) was identified as [2-bromomaleylacetate-H][−] with peaks at *m/z* 234.9245 (with ⁷⁹Br) and *m/z* 236.9223 (with ⁸¹Br) and its first fragment under ionization conditions at *m/z* 190.9338 (loss of —COO[−] from 234.9245) and *m/z* 190.9326 (loss of —COO[−] from 236.9223) (Fig. 5B). Because Br has two stable isotopes (⁷⁹Br and ⁸¹Br) with nearly equal natural abundances, peaks at *m/z* 234.9245 and *m/z* 236.9223 and a relative intensity ratio of 1:1 are the characteristic of a molecule with one bromine atom. No 2-bromomaleylacetate was found in the control using *E. coli* DH5α(pUC18). All these data showed that OdcB is a dioxygenase that converts 2,6-DBHQ to 2-bromomaleylacetate.

TABLE 3 Substrate spectrum of OdcA

Substrate	Activity (nmol min ^{−1} mg ^{−1})
3,5-Dibromo-4-hydroxybenzoate	1,321.4 ± 38.4
3-Bromo-4-hydroxybenzoate	92.2 ± 4.5
3,5-Dichloro-4-hydroxybenzoate	944.2 ± 38.2
3-Chloro-4-hydroxybenzoate	81.8 ± 14.8
4-Hydroxybenzoate	— ^a
3,5-Dibromobenzoate	—
4-Nitrophenol	—
2-Chloro-4-nitrophenol	—
2,6-Dichloro-4-nitrophenol	—
2,4,6-Trichlorophenol	—
3,5-Dichloro-4-hydroxyaniline	—
3,5-Dibromo-4-hydroxybenzonitrile	—
6-Hydroxy-3-succinoylpyridine	—

^a—, no enzyme activity was detected.

OdcC converts 2-bromomaleylacetate to β -ketoadipate. The end metabolite produced from DBHB by *E. coli* DH5 α (pUC-odcABC) was first trimethylsilylated and showed a retention time of 18.1 min in the GC trace (Fig. 5C), which matched β -ketoadipate in the NIST library with 86% probability. The end metabolite, which showed a molecular ion peak at m/z 376 and fragments at m/z 361 (loss of $-\text{CH}_3$), m/z 286 (loss of six $-\text{CH}_3$), m/z 259 [loss of $-\text{COOSi}(\text{CH}_3)_3$], m/z 243 [loss of $-\text{Si}(\text{CH}_3)_3$ and $-(\text{CH}_3)_4$], m/z 231 [loss of $-\text{CH}_2\text{CH}_2\text{COOSi}(\text{CH}_3)_3$], m/z 125 [loss of $-\text{Si}(\text{CH}_3)_3$ and two $-\text{OSi}(\text{CH}_3)_3$], and m/z 73 [$-\text{Si}(\text{CH}_3)_3$] by MS, was identified as trimethylsilylated β -ketoadipate. In the control using *E. coli* DH5 α (pUC-odcAB), no β -ketoadipate was found. Based on the above results, OdcC was confirmed to be capable of converting 2-bromomaleylacetate to β -ketoadipate.

DISCUSSION

In addition to the previously reported anaerobic reductive dehalogenation pathway and the aerobic reductive dehalogenation plus oxidative ring cleavage pathway, a new oxidative decarboxylation pathway for DBHB catabolism was found in strain H8 in this study (Fig. 1C and D). DBHB is initially converted to 2,6-DBHQ via an oxidative decarboxylation process catalyzed by the NAD(P)H-dependent flavin monooxygenase OdcA (Orf420); then, 2,6-DBHQ is ring cleaved to 2-bromomaleylacetate by the dioxygenase OdcB (Orf425). Finally, 2-bromomaleylacetate is reduced to β -ketoadipate by the maleylacetate reductase OdcC (Orf426). This third pathway for DBHB catabolism deepens our understanding of the catabolic diversity of halogenated hydroxybenzoates.

Flavin monooxygenases play key roles in the initial degradation of hydroxybenzoates and their derivatives (HBDs) in aerobes. Flavin monooxygenases catalyze the hydroxylation of HBDs via the addition of one of the atoms from O_2 to the aromatic ring, with consumption of an equimolar amount of NADPH or NADH (serving as electron donor), yielding dihydroxybenzoate compounds or dihydroxy-benzene compounds (decarboxylative hydroxylation). Interestingly, decarboxylative hydroxylation reactions catalyzed by flavin monooxygenases are significantly affected by the hydroxyl group position, i.e., *ortho*-, *meta*-, or *para* to the carboxyl group (28). For *ortho*-HBDs (salicylate derivatives), decarboxylative hydroxylation (1-hydroxylation reaction) appears to be common, and many salicylate 1-monooxygenases (also called salicylate 1-hydroxylases) that convert salicylate derivatives to catechol derivatives have been characterized, including NahG (P23262) from *Pseudomonas putida* PpG7 (29), NahG (AAD02146) and NahW (Q9Z164) from *Pseudomonas stutzeri* AN10 (30, 31), SalA (Q9RB12) from *Acinetobacter* sp. strain ADP1 (32), SalA (Q9HFQ8) from *Aspergillus nidulans* (33), and NahG (AAP44249 and AAP44222) from *Pseudomonas* sp. strain ND6 (34). To our knowledge, no direct decarboxylative hydroxylation of *meta*-HBDs to resorcinol derivatives has been reported. For *para*-HBDs, several 1-hydroxylases/monooxygenases have been identified in fungi, e.g., vanillate hydroxylase (amino acid sequence unknown) from *Phanerochaete chrysosporium* (35), *para*-hydroxybenzoate 1-hydroxylases from *Candida parapsilosis* CBS604 (amino acid sequence unknown) (36) and *Candida parapsilosis* CDC317 (MNX1, G8B709) (37), and prenyl *para*-hydroxybenzoate 1-monooxygenase (VibMO1, ANB32222) from *Boreostereum vibrans* (38). However, to date, no 1-monooxygenase/hydroxylase for *para*-HBDs has been identified in prokaryotes. The OdcA identified in our *Pigmentiphaga* sp. strain H8 represents a novel 1-monooxygenase for *para*-HBDs found in prokaryotes; we determined that the *para* hydroxyl group is necessary for the substrate to be decarboxylated by OdcA (3,5-dibromobenzoate was not transformed by OdcA). The lack of detectable activity of OdcA against 4-HB is slightly perplexing. It is possible that the halogen *ortho* to the hydroxyl group is also important for substrate binding because DBHB (K_m , 32.4 μM) had a lower K_m than did BHB (K_m , 187.9 μM).

Flavin monooxygenases constitute a large family of flavoenzymes, comprising at least 130 members (classified into eight groups, A to H) first characterized starting in 1957, that catalyze hydroxylation, Baeyer-Villiger oxidation, sulfoxidation, epoxidation, and halogenation reactions (27). Sequence alignment and phylogenetic analysis show that OdcA belongs to the group A flavin monooxygenases (Fig. 3). Compared to other

FIG 6 Sequence alignment of OdcA with closely related proteins available in UniProtKB and the Protein Data Bank. PnpA (C1I201), *para*-nitrophenol 4-hydroxylase from *Pseudomonas* sp. strain WBC-3; HspB (F8G0M4), 6-hydroxy-3-succinoylpyridine 3-hydroxylase from *P. putida* S16; MNX1 (G8B709), *para*-hydroxybenzoate 1-hydroxylase from *C. parapsilosis* CDC317; VibMO1 (ANB32222), *para*-hydroxybenzoate 1-hydroxylase from *Boreostereum vibrans*; Sal (Q59713), salicylate 1-hydroxylase from *P. putida* S-1; SalA (Q9RB12), salicylate 1-hydroxylase from *Acinetobacter* sp. strain ADP1; NahG (P23262), salicylate 1-hydroxylase from *P. putida* PpG7; NahW (Q9Z164), salicylate 1-hydroxylase from *P. stutzeri* AN10; MtmOIV (4K55_A), Baeyer-Villiger monooxygenase from *Streptomyces argillaceus*; RIFMO (5KOW_A), *N*-hydroxylating rifampin monooxygenase from *Nocardia farcinica*; CabE (2QA2_A), aromatic hydroxylase from *Streptomyces* sp. strain H021; OxyS (4K2X_A), anhydrotetracycline hydroxylase from *Streptomyces rimosus*. The red asterisks indicate residues that participate in hydrogen bonding with FAD. The red triangles indicate residues potentially important for NADPH binding. Conserved motifs [PxGG and GxGxxG(x)₁₇E] are underlined.

flavin monooxygenase members in group A, OdcA exhibits certain interesting features. First, OdcA shows the highest sequence identities to PnpA (36%) and HspB (34%), which catalyze the hydroxylation of a nitro or succinoyl group *para* to an existing hydroxyl group of nonhydroxybenzoate substrates (e.g., *para*-nitrophenol and 6-hydroxy-3-succinoylpyridine) (18, 19). OdcA demonstrated relatively low sequence identities with functionally similar hydroxybenzoate decarboxylases, e.g., *para*-hydroxybenzoate 1-hydroxylases (MNX1 and VibMO1) and *ortho*-hydroxybenzoate 1-hydroxylases (Sal, SalA, NahG, and NahW) (28–31). Second, sequence alignment of OdcA with PnpA, HspB, MNX1, VibMO1, SalA, NahG, NahW, and certain closely related monooxygenases with reported crystal structures (MtmOIV, 4K55_A; OxyS, 4K2X_A; CabE, 2QA2_A; and RIFMO, 5KOW_A) demonstrated that OdcA is comprised of only two domains, the FAD-binding domain (residues 1 to 181 and 264 to 385) and the middle domain (residues 182 to 263), and lacks the C-terminal thioresin-like domain (Fig. 6) (39–42). Third, among the six residues (E34, Q107, V132, D292, L305, and N306) in OdcA proposed to participate in hydrogen bonding with FAD (39, 40), three residues (Q107, V132, and L305) were not conserved in the five decarboxylases with similar function (MNX1, VibMO1, SalA, NahG,

TABLE 4 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Source or reference
Strains		
<i>Pigmentiphaga</i> sp.		
H8	DBHB-degrading strain, wild type; Spe ^r Str ^r	This study
H8Δ <i>odcA</i>	Mutant of strain H8 with <i>odcA</i> gene deleted	This study
H8Δ <i>odcA</i> (pMCS- <i>odcA</i>)	Mutant H8Δ <i>odcA</i> complemented with <i>odcA</i> gene by harboring the plasmid pMCS- <i>odcA</i>	This study
<i>E. coli</i>		
DH5α	Host strain for cloning vectors	TaKaRa
DH5α λpir	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 deoR Δ(lacZYA-argF)U169 λpir⁺</i>	Lab stored
HB101(pRK2013)	Conjugation helper strain; Km ^r	Lab stored
BL21(DE3)	Host strain for expression vectors	TaKaRa
Plasmids		
pBBR1MCS-2	Broad-host-range vector; Km ^r	59
pMCS- <i>odcA</i>	<i>odcA</i> gene with its own promoter inserted into pBBR1MCS-2; Km ^r	This study
pUC18	Cloning and expression vector; Amp ^r	TaKaRa
pUC- <i>odcA</i>	Codon-optimized <i>odcA</i> gene inserted into pUC18; Amp ^r	This study
pUC- <i>odcB</i>	<i>odcB</i> gene inserted into pUC18; Amp ^r	This study
pUC- <i>odcAB</i>	<i>odcB</i> gene inserted into pUC- <i>odcA</i> ; Amp ^r	This study
pUC- <i>odcABC</i>	<i>odcC</i> gene inserted into pUC- <i>odcAB</i> ; Amp ^r	This study
pJQ200SK	Suicide vector; P15A <i>ori sacB</i> RP4 (pBluescriptSK); Gm ^r	60
pJQ-Δ <i>odcA</i>	pJQ200SK containing the upstream and downstream fragments of <i>odcA</i> gene for gene targeting of <i>odcA</i> gene; Gm ^r	This study
pET29a(+)	Expression vector; Km ^r	Lab stored
pET- <i>odcA</i>	Codon-optimized <i>odcA</i> gene inserted into pET29a(+); Km ^r	This study

^aSpe^r, spectinomycin resistance; Km^r, kanamycin resistance; Gm^r, gentamicin resistance; Amp^r, ampicillin resistance; Str^r, streptomycin resistance.

and NahW). The residue N306 was identified only in NahW. Fourth, the conserved patch P299XGG302 in *OdcA*, which helps to position the substrate with respect to the FAD isoalloxazine ring (40), was not identified in the five decarboxylases with similar function. Fifth, among the four arginine residues (R169, R173, R174, and R277) in MtmOIV that are potentially important for binding NADPH, which were revealed by low-resolution electron density mapping (41), three (R168, R172, and R277) were identified in *OdcA*, and only one or two were identified in the five decarboxylases with similar function. The distinct features of *OdcA* revealed by sequence analysis might explain, to some extent, the special substrate specificity and cofactor type of *OdcA*.

In general, the discovery of a new oxidative decarboxylation pathway for DBHB catabolism at the molecular level deepens our understanding of the mechanism underlying the catabolic diversity of DBHB, and the characterization of the novel DBHB 1-monooxygenase *OdcA* in the group A flavin monooxygenase family provides insights into the hydroxylation of the carboxyl group *para* to an existing hydroxyl group on HBDs.

MATERIALS AND METHODS

Chemicals, bacterial strains, plasmids, primers, and culture conditions. The standard compounds DBHB, BHB, 4-HB, DCHB, CHB, 3,5-dibromobenzoate, 2,6-dichloro-4-nitrophenol, 2-chloro-4-nitrophenol, 4-nitrophenol, 2,4,6-trichlorophenol, 2,6-dichloro-4-aminophenol, and 3,5-dibromo-4-hydroxybenzonitrile were purchased from Energy Chemical Co., Ltd. (Shanghai, China) or J&K Chemical Co., Ltd. (Shanghai, China). The purities of all standard compounds exceeded 98%. 2,6-DBHQ was obtained from the transformation of DBHB by *OdcA*. High-performance liquid chromatography (HPLC)-grade methanol and dichloromethane were purchased from Merck Co., Ltd. (Darmstadt, Germany). All other reagents used in this study were of analytical grade.

The bacterial strains and plasmids used in this study are listed in Table 4, and the primers used in this study are described in Table 5. *Pigmentiphaga* sp. strain H8, which is capable of utilizing DBHB as its sole source of carbon and energy for growth, was isolated previously from soil contaminated with halogenated aromatic compounds using a conventional enrichment culture technique. This strain has been deposited in the China Center for Type Culture Collection (CCTCC) under accession number M 2017222.

E. coli and *Pigmentiphaga* strains were grown aerobically at 37°C in lysogeny broth (LB). For the degradation assay, MSM (1.0 g of NH₄NO₃, 1.6 g of K₂HPO₄, 0.5 g of KH₂PO₄, 0.2 g of MgSO₄, and 1.0 g of NaCl per liter of water, pH 7.0) supplemented with 0.2 mM DBHB was used. To measure bromide release from DBHB, NaCl was removed from the MSM to avoid interference. Antibiotics were added as

TABLE 5 Primers used in this study

Primers	Sequence, 5'→3' ^a (restriction enzyme site)	Purpose
odcA29-f	5'-CGACATATG TGGAGCCACCCGAGTTCGAAAG ACCAGTGAAACCCAGGTG-3' (NdeI)	Amplification of codon-optimized <i>odcA</i> gene for cloning into pET29a(+) vector
odcA29-r	5'-CTAAAGCTTTTACGGAATGGCTG-3' (HindIII)	
Kn1	5'-GCAGGATCCACTCAGGTAGTATCGTTGG-3' (BamHI)	Amplification of 510-bp fragment of <i>odcA</i> (positions 13–522) for gene knockout
Kn2	5'-ATGCGCCACAGGTTCCCGACGCTCTTCTGACCGCGCT-3'	
Kn3	5'-GCAGCGCGGTGAGGAAGAGCGTCGGGAACCTGTGGCGC-3'	Amplification of 504-bp fragment of <i>odcA</i> (positions 673–1176) for gene knockout
Kn4	5'-CTACTGCAGGATCATGGAAGTGCAGCGCA-3' (PstI)	
odcA-mcs-f	5'-CGAAAGCTTGCATGCGCGAACGCTATGC-3' (BamHI)	Amplification of 3,883-bp fragment (including <i>odcA</i> , <i>orf421</i> , and <i>orf422</i>) for gene complementation
odcA-mcs-r	5'-CATCTAGATAGCGGCGCCTTACGCCCCCT-3' (XbaI)	
odcA-pUC-f	5'-GCAGGTACCTAAGAAGGAGATATACATATGACCAAGTAAACCC-3' (KpnI)	Amplification of codon-optimized <i>odcA</i> gene for cloning into pUC18 vector
odcA-pUC-r	5'-GCAGGATCCTTAGCGAATGGCTGCTGC-3' (BamHI)	
odcB-puc-f	5'-GCAGGTACCAACACACAGGAGACGACATG-3' (KpnI)	Amplification of <i>odcB</i> for cloning into pUC18 vector
odcB-puc-r	5'-GCAGGATCCTCAGAGGTGGATGGGATCT-3' (BamHI)	
odcB-puc-f2	5'-GCAGGATCCACACACAGGAGACGACATG-3' (BamHI)	Amplification of <i>odcB</i> for cloning into pUC- <i>odcA</i> vector
odcB-puc-r2	5'-GCAGTCACTCAGAGGTGGATGGGATCT-3' (SalI)	
odcC-puc-f	5'-GCAGTCACTCAGAGGTGGATGGGATCT-3' (SalI)	Amplification of <i>odcC</i> for cloning into pUC- <i>odcAB</i> vector
odcC-puc-r	5'-CATAAGCTTCTACTGCGCGCTAACGGCGCG-3' (HindIII)	

^aThe restriction enzyme sites are underlined, and the nucleotides that encode the Strep-II tag are in bold.

necessary at the following concentrations: ampicillin (Amp), 100 mg liter⁻¹; kanamycin (Km), 50 mg liter⁻¹; spectinomycin (Spe), 80 mg liter⁻¹; and gentamicin (Gm), 100 mg liter⁻¹.

Inducible-degradation assay. The inducible degradation of DBHB by strain H8 was performed as described previously (43) with some modifications. Half of the washed strain H8 cells (grown on LB) were inoculated into MSM with 0.2 mM DBHB and 2 mM glucose (induced cells), and the other half were inoculated into the MSM with 2 mM glucose only (uninduced cells). After incubation for 8 h, induced and uninduced cells were harvested, washed twice with MSM, inoculated individually into MSM (containing 0.2 mM DBHB) at an OD₆₀₀ of 0.3, and cultured in a shaker at 37°C. One milliliter of culture was sampled at regular intervals (3 h) and centrifuged at 16,000 × *g* at 4°C for 10 min. DBHB concentrations in the supernatants were determined by HPLC. The experiment was repeated four times.

Draft genome sequencing of strain H8. DNA manipulation was performed according to standard protocols (44). Approximately 33 μg of genomic DNA of strain H8 was delivered to Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China) for draft genome sequencing using an Illumina MiSeq system. The sequencing procedure, redundant sequence removal, reads assembly, *de novo* gene prediction, and functional annotation were performed as described previously (45).

Sequence analysis. The signal peptide was predicted using the SignalP 4.1 server (<http://www.cbs.dtu.dk/services/SignalP/>) (46). The phylogenetic tree for OdcA and its closely related proteins was constructed using the neighbor-joining method with 1,000 bootstrap replicates (47, 48). The related protein sequences retrieved from GenBank, UniProtKB, and the *Candida parapsilosis* genome database were aligned using Bioedit (v6.0.7) (49). The secondary structure of OdcA was predicted using neural network systems (PHD, https://npsa-prabi.ibcp.fr/NPSA/npsa_phd.html) (50).

Comparative transcriptome analysis (RNA-seq). Total RNA obtained from DBHB-induced and uninduced cells of strain H8, prepared as described above, was isolated using a MiniBEST universal RNA extraction kit (TaKaRa, Dalian), followed by DNase treatment (gDNA Eraser; TaKaRa, Dalian). The experiment was performed twice independently. The isolated RNA was delivered to Novogene Bioinformatics Technology Co., Ltd., Beijing, China, for reverse transcription and sequencing. Quantified cDNA libraries (effective concentration, >2 nM) were sequenced using an Illumina HiSeq platform. Clean reads were obtained by removing low-quality reads, and reads containing poly-N and adapters were mapped back onto the draft genome sequence of strain H8 using Bowtie2 (v2.2.3) (51). HTSeq v0.6.1 software was used to count the numbers of reads mapped to each gene (52). Transcript abundance was measured as a unit of the expected number of fragments per kilobase of transcript per million mapped reads (FPKM) (61). Differential expression analysis of two groups (DBHB-induced and uninduced cells) was performed using the DESeq R package (1.18.0). Genes with an adjusted *P* value of <0.05 found by DESeq were considered significantly differentially expressed. Gene ontology (GO) enrichment analysis of differentially expressed genes (DEGs) was performed using the GOSec R package (release 2.12) (53). The statistical enrichment of DEGs in KEGG pathways was performed using KOBAS v2.0 (<http://kobas.cbi.pku.edu.cn/>).

Gene knockout and complementation. For *odcA* gene knockout, upstream and downstream fragments of the *odcA* gene were amplified with the primer pairs Kn1-Kn2 (with BamHI at the 5' site) (Table 5) and Kn3-Kn4 (with PstI at the 3' site). The two fragments were spliced by overlapping PCR (54) and inserted into the BamHI and PstI sites of pJQ200SK to generate the recombinant plasmid pJQ-Δ*odcA*. The plasmid pJQ-Δ*odcA* was introduced into strain H8 via triparental mating with the helper strain *E. coli* HB101 (pRK2013). Single-crossover recombinants were screened on LB plates supplemented with 80 mg liter⁻¹ Spe and 100 mg liter⁻¹ Gm and cultured on LB agar containing 8% (wt/vol) sucrose. After 2 days of growth at 37°C, individual colonies were plated on Gm-containing and Spe-containing LB plates. Finally, double-crossover recombinants were screened on 8% sucrose LB plates, and the *odcA* deletion mutant H8Δ*odcA* was selected and confirmed by PCR analysis.

To perform *odcA* gene complementation in the mutant H8 Δ *odcA*, the *odcA* gene (including its native promoter) was inserted into BamHI-XbaI-digested pBBR1MCS2 to generate pMCS-*odcA*. The plasmid pMCS-*odcA* was transformed into the mutant H8 Δ *odcA* via triparental mating, and the complemented strain was screened on LB plates supplemented with 80 mg liter⁻¹ Spe and 50 mg liter⁻¹ Km. The mutant H8 Δ *odcA* harboring the empty plasmid pBBR1MCS-2 was also used as the negative control.

Protein expression and purification. The codon-optimized *odcA* gene (see the supplemental material) was amplified with the primer pair *odcA*29-f/*odcA*29-r (Table 5) and cloned into the NdeI and HindIII sites of pET29a(+) to produce pET-*odcA*. The sequence was verified by DNA sequencing to ensure that no mutations were introduced. *E. coli* BL21(DE3) harboring pET-*odcA* was grown in LB at 37°C to an OD₆₀₀ of 0.6 and induced for 18 h via the addition of 0.3 mM isopropyl- β -D-thiogalactopyranoside at 16°C. The cells were harvested by centrifugation, suspended in binding buffer (100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 8.0), and disrupted by sonication. After centrifugation at 16,000 $\times$ g for 15 min at 4°C, the Strep II-tagged protein was purified using a Strep-Tactin Sepharose chromatograph. The supernatant was charged onto the column containing Strep-Tactin Sepharose HP (GE Healthcare Life Sciences) and washed with 10 ml of binding buffer. The target protein was eluted with 5 ml of elution buffer (100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 2.5 mM dethiobiotin, pH 8.0).

The eluted protein was dialyzed in one liter of 20 mM Tris-HCl (pH 7.5) overnight to remove EDTA and dethiobiotin. The purity of the purified protein was detected using SDS-PAGE with a 12% polyacrylamide resolving gel and a 5% stacking gel. The protein concentration was determined by the Bradford method (62) using bovine serum albumin as a standard.

Enzymatic assay for OdcA. To perform UV spectrum scanning of DBHB transformation by OdcA, sample and reference cuvettes contained 0.04 mM NADPH, 0.002 mM FAD, 20 mM Tris-HCl buffer (pH 7.5), and 0.05 μ M purified Strep-OdcA in a 1-ml volume, and reactions were initiated by the addition of 0.03 mM DBHB. To perform a standard enzyme assay for OdcA, the reaction system (1.0 ml) contained 0.3 mM DBHB, 0.3 mM NADPH, 0.13 μ M purified Strep-OdcA, and 20 mM Tris-HCl buffer (pH 7.5). The reaction mixture was incubated at 35°C for 10 min, and the reaction was stopped by heating. The mixture was centrifuged at 16,000 $\times$ g for 10 min and filtered via membrane filtration (pore size, 0.22 μ m), and the residual DBHB was analyzed by HPLC.

To investigate the effects of cofactors on OdcA activity, different cofactors (e.g., 0.3 mM NADPH, 0.3 mM NADH, 0.01 mM FAD, or 0.01 mM flavin mononucleotide [FMN]) were added to 1.0 ml of a 20 mM Tris-HCl buffer reaction system (pH 7.5) containing 0.3 mM DBHB and 0.13 μ M OdcA. The relative activity of OdcA was calculated compared to the activity of the standard enzyme assay (with 0.3 mM NADPH as the cofactor). The optimum temperature and pH value for OdcA activity were determined in the standard enzyme reaction system at different temperatures (4 to 45°C) or pH values (3.5 and 10.0) using different buffers. To detect the activity of OdcA under anaerobic conditions, the enzyme reaction was performed in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) with 0.26 μ M OdcA at 35°C. All enzyme assays were repeated independently three times, and the means and standard deviations were calculated.

The Michaelis-Menten kinetics of the oxidative decarboxylation catalyzed by Strep-OdcA were determined by plotting reaction rates against seven concentrations of DBHB (10 to 100 μ M) in the presence of 200 μ M NADPH, 10 μ M FAD, 0.05 μ M Strep-OdcA, and 20 mM Tris-HCl buffer (pH 7.5) or against BHB (60 to 280 μ M) in the presence of 200 μ M NADPH, 10 μ M FAD, 0.15 μ M Strep-OdcA, and 20 mM Tris-HCl buffer (pH 7.5). Kinetic data were evaluated via nonlinear regression analysis with the Michaelis-Menten equation. To measure enzyme kinetics, the consumption of NADPH (λ_{max} , 340 nm) was used to determine oxidoreductase activity, and the molar extinction coefficient for NADPH was 6,220 M⁻¹ cm⁻¹ at 340 nm (55). One unit of enzyme activity was defined as the amount of enzyme that catalyzes the consumption of 1 μ mol NADPH per minute at 35°C. The specific enzyme activity was expressed in enzyme activity units per milligram of protein.

Functional identification of OdcB. High-level expression of OdcB with a tag in *E. coli* BL21(DE3) failed, and no activity was detected for the purified OdcB. To identify the function of OdcB, the *odcB* gene was amplified using the primer pair *odcB*-puc-f/*odcB*-puc-r (Table 5) and inserted into KpnI/BamHI-digested pUC18 to generate pUC-*odcB*. The constructed plasmid was transformed into *E. coli* DH5 α to obtain *E. coli* DH5 α (pUC-*odcB*). The substrate 2,6-DBHQ was prepared and purified from the conversion of DBHB by OdcA. The transformation of 2,6-DBHQ by *E. coli* DH5 α (pUC-*odcB*) was monitored regularly by UV spectrum scanning. *E. coli* DH5 α carrying the empty pUC18 vector was used as the negative control. Briefly, *E. coli* cells were inoculated into MSM supplemented with 0.1 mM 2,6-DBHQ and 2 mM glucose at a final OD₆₀₀ of 1.0. At regular intervals (1 h), 1 ml of the culture was sampled and centrifuged at 16,000 $\times$ g at 4°C for 2 min, and then the supernatant was added to 1 mM NaBH₄ to reduce 2,6-dibromobenzoquinone to 2,6-DBHQ before UV scanning (20). To detect the transformation of 2,6-DBHQ by *E. coli* DH5 α (pUC-*odcB*) under anaerobic conditions, the whole-cell transformation experiment mentioned above was performed in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI).

Functional identification of OdcC. To identify the function of OdcC, the *odcA*, *odcB*, and *odcC* genes were amplified using the primer pairs *odcA*-puc-f/*odcA*-puc-r, *odcB*-puc-f2/*odcB*-puc-r2, and *odcC*-puc-f/*odcC*-puc-r (Table 5) and then inserted into pUC18 one by one to generate pUC-*odcA*, pUC-*odcAB*, and pUC-*odcABC*, respectively. The constructed plasmids were transformed into *E. coli* DH5 α to obtain *E. coli* DH5 α (pUC-*odcA*), *E. coli* DH5 α (pUC-*odcAB*), and *E. coli* DH5 α (pUC-*odcABC*). *E. coli* DH5 α (pUC-*odcABC*) was inoculated into 10 ml of MSM supplemented with 0.1 mM DBHB and 2 mM glucose at a final OD₆₀₀ of 1.0. After 8 h, the end metabolite produced was identified compared to that produced by *E. coli* DH5 α (pUC-*odcAB*).

Determination of DBHB, 2,6-DBHQ, and bromine ion. DBHB and its decarboxylation product 2,6-DBHQ were qualitatively and quantitatively determined using a Dionex UltiMate 3000SD HPLC system equipped with a diode array detector (DAD) and a C₁₈ reversed-phase column (4.6 by 250 mm, 5- μ m particle size; Dionex). The mobile phase was 60% methanol containing 0.5% acetic acid, and the flow rate was 1.0 ml min⁻¹. The column temperature was 30°C, and the injection volume was 20 μ l. DBHB and 2,6-DBHQ were detected at 250 nm and 298 nm, respectively. Ascorbic acid was used to reduce dibromo-*p*-benzoquinone to 2,6-DBHQ before the qualitative and quantitative detection of 2,6-DBHQ by HPLC (20). Under these conditions, the retention times for DBHB and 2,6-DBHQ by HPLC were 9.2 min and 5.8 min, respectively. The detection limits for DBHB and 2,6-DBHQ under these conditions are 0.5 μ M and 5 μ M, respectively. The bromine ion released during DBHB catabolism was detected and quantified according to the method described by Bergmann and Sainik (56).

Identification of metabolites. The metabolite produced from DBHB by OdcA was identified by GC-MS (450-GC, 320-MS; Bruker) equipped with a BR-5ms capillary column (30 m by 0.25 mm by 0.25 μ m). The metabolite was extracted and derivatized using a previously described method (57). Briefly, the reaction solution (10 ml) was acidified to pH 1 (by adding 80 μ l of 5 M HCl), and the aromatic metabolite was extracted with an equal volume of dichloromethane. The organic phase was dried under N₂ flow, and the remaining solid was dissolved in a 200- μ l mixture of pyridine and acetic anhydride (1:3, vol/vol). The solution was heated at 45°C for 20 min and analyzed by GC-MS at a flow rate of 1 ml min⁻¹ of helium. The column temperature began at 50°C for 1 min, increased to 210°C at 10°C min⁻¹, was held for 2 min, increased to 240°C at 20°C min⁻¹, and was held for 10 min. The injector temperature was set to 250°C with a split ratio of 30:1. Both the interface temperature and ion source temperature were set to 230°C. The column outlet was inserted directly into the electron ionization source block and operated at 70 eV. The sample was analyzed with a scan interval of 0.34 s and an *m/z* range of 50 to 600. The intermediates were identified using an NIST MS data library based on comparisons of GC retention times and mass spectra with those of authentic compounds.

The metabolite produced from 2,6-DBHQ by *E. coli* DH5 α (pUC-odcB) was identified by an LC-MS system (G2-XS QToF; Waters) as previously described with some modifications (20). Briefly, 20 ml of reaction culture was centrifuged at 16,000 $\times g$ at 4°C for 5 min, and the supernatant was acidified to pH 1 by adding 5 M HCl and extracted with an equal volume of ethyl acetate. The organic phase was dried over Na₂SO₄ before evaporation under N₂ flow. Extracts were redissolved in 1 ml methanol, and then a 2- μ l sample was injected into an ultraperformance liquid chromatography (UPLC) column (2.1 by 100 mm; Acquity UPLC BEH C₁₈ column containing 1.7- μ m particles) with a flow rate of 0.4 ml min⁻¹. The mobile phase consisted of buffer A (0.1% formic acid in water) and buffer B (0.1% formic acid in acetonitrile) with a gradient program of 2% buffer B for 0.5 min, 2 to 20% buffer B for 5 min, an increase to 95% buffer B for 6 min, and 95% buffer B for 2 min. Mass spectrometry was performed using an electrospray source in negative ion mode with MS^e acquisition mode, with a selected mass range of 50 to 600 *m/z*. The ionization parameters were as follows: capillary voltage, 2.5 kV; collision energy, 40 eV; source temperature, 120°C; and desolvation gas temperature, 400°C. Data acquisition and processing were performed using Masslynx 4.1. Under these conditions, the metabolite was detected by the mass detector at 2.2 min.

The end metabolite produced from DBHB by *E. coli* DH5 α (pUC-odcABC) was identified by a GC-MS system. The reaction culture was centrifuged at 16,000 $\times g$ at 4°C for 5 min, and the supernatant was acidified to pH 1 by adding 5 M HCl and extracted with an equal volume of ethyl acetate. The organic phase was dried over Na₂SO₄ before evaporation under N₂ flow, and the metabolite was derivatized using a previously described method with minor modifications (58). Briefly, the metabolite was dissolved in a 200- μ l mixture of acetonitrile and *N,O*-bis(trimethylsilyl)trifluoroacetamide (95:5, vol/vol). The solution was heated at 75°C for 30 min and analyzed by GC-MS (QP2010; Shimadzu) with an RTX-5ms capillary column (30 m by 0.25 mm by 0.25 μ m) at a flow rate of 1.22 ml min⁻¹ of helium. The column temperature began at 50°C for 3 min, increased to 210°C at 10°C min⁻¹, was held for 2 min, increased to 240°C at 20°C min⁻¹, and was held for 10 min. The injector temperature was set to 250°C with a split ratio of 30:1. Both the interface temperature and ion source temperature were set to 230°C. The column outlet was inserted directly into the electron ionization source block and operated at 70 eV. The sample was analyzed with a scan interval of 0.5 s and an *m/z* range of 50 to 600. The metabolite was identified using an NIST MS data library based on comparisons of GC retention times and mass spectra with those of authentic compounds.

Accession number(s). The nucleotide sequences of the 16S rRNA gene and the DBHB catabolic gene cluster (*orf420* to *orf426*) of *Pigmentiphaga* sp. strain H8 have been deposited in the GenBank database under accession numbers [MF193477](#) and [MF197311](#), respectively.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.02467-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

This work was financially supported by grants from the National Natural Science Foundation of China (31400105), the National Key Research and Development Program of China (2016YFD0800203), the China Postdoctoral Science Foundation (2015T80562

and 2014M561666), the joint NSFC-ISF (31461143009), and the Fundamental Research Funds for the Central Universities (KYZ201742).

REFERENCES

- Ni S, Fredrickson JK, Xun L. 1995. Purification and characterization of a novel 3-chlorobenzoate-reductive dehalogenase from the cytoplasmic membrane of *Desulfomonile tiedjei* DCB-1. *J Bacteriol* 177:5135–5139. <https://doi.org/10.1128/jb.177.17.5135-5139.1995>.
- Smidt H, de Vos WM. 2004. Anaerobic microbial dehalogenation. *Annu Rev Microbiol* 58:43–73. <https://doi.org/10.1146/annurev.micro.58.030603.123600>.
- Wagner A, Cooper M, Ferdi S, Seifert J, Adrian L. 2012. Growth of *Dehalococcoides mccartyi* strain CBDB1 by reductive dehalogenation of brominated benzenes to benzene. *Environ Sci Technol* 46:8960–8968. <https://doi.org/10.1021/es3003519>.
- Golovleva LA, Pertsova RN, Kunc F, Vokounová M. 1988. Decomposition of the herbicide bromoxynil in soil and in bacterial cultures. *Folia Microbiol (Praha)* 33:491–499. <https://doi.org/10.1007/BF02925776>.
- Stalker DM, McBride KE. 1987. Cloning and expression in *Escherichia coli* of a *Klebsiella ozaenae* plasmid-borne gene encoding a nitrilase specific for the herbicide bromoxynil. *J Bacteriol* 169:955–960. <https://doi.org/10.1128/jb.169.3.955-960.1987>.
- Holtze MS, Sørensen SR, Sørensen J, Aamand J. 2008. Microbial degradation of the benzonitrile herbicides dichlobenil, bromoxynil and ioxynil in soil and subsurface environments—insights into degradation pathways, persistent metabolites and involved degrader organisms. *Environ Pollut* 154:155–168. <https://doi.org/10.1016/j.envpol.2007.09.020>.
- Székács A, Darvas B. 2012. Environmental and ecological aspects of first generation genetically modified crops regarding their impacts in a European maize producer country. *IJEP* 2:9–15.
- Eberlein CV, Guttieri MJ, Steffen-Campbell J. 1998. Bromoxynil resistance in transgenic potato clones expressing the *bxn* gene. *Weed Sci* 46:150–157.
- Liu X, Li X, Gao L, Cui C, Li C, Li J, Wang B. 2011. Extraction and PTP1B inhibitory activity of bromophenols from the marine red alga *Symphocladia latiuscula*. *Chin J Oceanol Limnol* 29:686–690. <https://doi.org/10.1007/s00343-011-0136-1>.
- Flodin C, Whitfield FB. 1999. 4-Hydroxybenzoic acid: a likely precursor of 2,4,6-tribromophenol in *Ulva lactuca*. *Phytochemistry* 51:249–255. [https://doi.org/10.1016/S0031-9422\(98\)00754-7](https://doi.org/10.1016/S0031-9422(98)00754-7).
- Gribble GW. 2010. Naturally occurring organohalogen compounds—a comprehensive update. Springer, Vienna, Austria.
- Veselá AB, Franc M, Pelantová H, Kubáč D, Vejvoda V, Šulc M, Bhalla TC, Macková M, Lovecká P, Janů P, Demnerová K, Martinková L. 2010. Hydrolysis of benzonitrile herbicides by soil actinobacteria and metabolite toxicity. *Biodegradation* 21:761–770. <https://doi.org/10.1007/s10532-010-9341-4>.
- Chen K, Huang L, Xu C, Liu X, He J, Zinder SH, Li S, Jiang J. 2013. Molecular characterization of the enzymes involved in the degradation of a brominated aromatic herbicide. *Mol Microbiol* 89:1121–1139. <https://doi.org/10.1111/mmi.12332>.
- Cupples AM, Sanford RA, Sims GK. 2005. Dehalogenation of the herbicides bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) and ioxynil (3,5-diiodo-4-hydroxybenzonitrile) by *Desulfotobacterium chlororespirans*. *Appl Environ Microbiol* 71:3741–3746. <https://doi.org/10.1128/AEM.71.7.3741-3746.2005>.
- Chen K, Jian S, Huang L, Ruan Z, Li S, Jiang J. 2015. Reductive dehalogenation of 3, 5-dibromo-4-hydroxybenzoate by an aerobic strain of *Delftia* sp. EOB-17. *Biotechnol Lett* 37:2395–2401. <https://doi.org/10.1007/s10529-015-1932-z>.
- Chen K, Xu X, Zhang L, Gou Z, Li S, Freilich S, Jiang J. 2015. Comparison of four *Comamonas* catabolic plasmids reveals the evolution of pBHB to catabolize haloaromatics. *Appl Environ Microbiol* 82:1401–1411. <https://doi.org/10.1128/AEM.02930-15>.
- Schmidt S, Kirby GW. 2001. Dioxigenative cleavage of C-methylated hydroquinones and 2, 6-dichlorohydroquinone by *Pseudomonas* sp. HH35. *Biochim Biophys Acta* 1568:83–89. [https://doi.org/10.1016/S0304-4165\(01\)00204-5](https://doi.org/10.1016/S0304-4165(01)00204-5).
- Zhang JJ, Liu H, Xiao Y, Zhang XE, Zhou NY. 2009. Identification and characterization of catabolic *para*-nitrophenol 4-monooxygenase and *para*-benzoquinone reductase from *Pseudomonas* sp. strain WBC-3. *J Bacteriol* 191:2703–2710. <https://doi.org/10.1128/JB.01566-08>.
- Tang H, Yao Y, Zhang D, Meng X, Wang L, Yu H, Ma L, Xu P. 2011. A novel NADH-dependent and FAD-containing hydroxylase is crucial for nicotine degradation by *Pseudomonas putida*. *J Biol Chem* 286:39179–39187. <https://doi.org/10.1074/jbc.M111.283929>.
- Xun L, Bohuslavsk J, Cai M. 1999. Characterization of 2, 6-dichloro-*p*-hydroquinone 1, 2-dioxygenase (PcpA) of *Sphingomonas chlorophenolica* ATCC 39723. *Biochem Biophys Res Commun* 266:322–325. <https://doi.org/10.1006/bbrc.1999.1805>.
- Xu L, Resing K, Lawson SL, Babbitt PC, Copley SD. 1999. Evidence that *pcpA* encodes 2,6-dichlorohydroquinone dioxygenase, the ring cleavage enzyme required for pentachlorophenol degradation in *Sphingomonas chlorophenolica* strain ATCC 39723. *Biochemistry* 38:7659–7669. <https://doi.org/10.1021/bi990103y>.
- Miyauchi K, Adachi Y, Nagata Y, Takagi M. 1999. Cloning and sequencing of a novel meta-cleavage dioxygenase gene whose product is involved in degradation of γ -hexachlorocyclohexane in *Sphingomonas paucimobilis*. *J Bacteriol* 181:6712–6719.
- Daubaras DL, Hershberger CD, Kitano K, Chakrabarty AM. 1995. Sequence analysis of a gene cluster involved in metabolism of 2,4,5-trichlorophenoxyacetic acid by *Burkholderia cepacia* AC1100. *Appl Environ Microbiol* 61:1279–1289.
- Seibert V, Thiel M, Hinner IS, Schlömann M. 2004. Characterization of a gene cluster encoding the maleylacetate reductase from *Ralstonia eutropha* 335^T, an enzyme recruited for growth with 4-fluorobenzoate. *Microbiology* 150:463–472. <https://doi.org/10.1099/mic.0.26602-0>.
- Schell MA, Sukordhaman M. 1989. Evidence that the transcription activator encoded by the *Pseudomonas putida nahR* gene is evolutionarily related to the transcription activators encoded by the *Rhizobium nodD* genes. *J Bacteriol* 171:1952–1959. <https://doi.org/10.1128/jb.171.4.1952-1959.1989>.
- Riebel A, Dudek HM, de Gonzalo G, Stepniak P, Rychlewski L, Fraaije MW. 2012. Expanding the set of rhodococcal Baeyer-Villiger monooxygenases by high-throughput cloning, expression and substrate screening. *Appl Microbiol Biotechnol* 95:1479–1489. <https://doi.org/10.1007/s00253-011-3823-0>.
- Huijbers MM, Montersino S, Westphal AH, Tischler D, van Berkel WJ. 2014. Flavin dependent monooxygenases. *Arch Biochem Biophys* 544:2–17. <https://doi.org/10.1016/j.abb.2013.12.005>.
- Uemura T, Kita A, Watanabe Y, Adachi M, Kuroki R, Morimoto Y. 2016. The catalytic mechanism of decarboxylative hydroxylation of salicylate hydroxylase revealed by crystal structure analysis at 2.5 Å resolution. *Biochem Biophys Res Commun* 469:158–163. <https://doi.org/10.1016/j.bbrc.2015.11.087>.
- You IS, Murray RI, Jollie D, Gunsalus IC. 1990. Purification and characterization of salicylate hydroxylase from *Pseudomonas putida* PpG7. *Biochem Biophys Res Commun* 169:1049–1054. [https://doi.org/10.1016/0006-291X\(90\)92000-P](https://doi.org/10.1016/0006-291X(90)92000-P).
- Bosch R, Moore ER, García-Valdés E, Pieper DH. 1999. NahW, a novel, inducible salicylate hydroxylase involved in mineralization of naphthalene by *Pseudomonas stutzeri* AN10. *J Bacteriol* 181:2315–2322.
- Bosch R, García-Valdés E, Moore ER. 2000. Complete nucleotide sequence and evolutionary significance of a chromosomally encoded naphthalene-degradation lower pathway from *Pseudomonas stutzeri* AN10. *Gene* 245:65–74. [https://doi.org/10.1016/S0378-1119\(00\)00038-X](https://doi.org/10.1016/S0378-1119(00)00038-X).
- Jones RM, Pagmantidis V, Williams PA. 2000. *sal* genes determining the catabolism of salicylate esters are part of a supraoperonic cluster of catabolic genes in *Acinetobacter* sp. strain ADP1. *J Bacteriol* 182:2018–2025. <https://doi.org/10.1128/JB.182.7.2018-2025.2000>.
- Graminha MA, Rocha EM, Prade RA, Martinez-Rossi NM. 2004. Terbinafine resistance mediated by salicylate 1-monooxygenase in *Aspergillus nidulans*. *Antimicrob Agents Chemother* 48:3530–3535. <https://doi.org/10.1128/AAC.48.9.3530-3535.2004>.
- Zhao H, Chen D, Li Y, Cai B. 2005. Overexpression, purification and characterization of a new salicylate hydroxylase from naphthalene-degrading *Pseudomonas* sp. strain ND6. *Microbiol Res* 160:307–313. <https://doi.org/10.1016/j.micres.2005.02.004>.
- Yajima Y, Enoki A, Mayfield MB, Gold MH. 1979. Vanillate hydroxylase

- from the white rot basidiomycete *Phanerochaete chrysosporium*. Arch Microbiol 123:319–321. <https://doi.org/10.1007/BF00406669>.
36. Eppink MH, Boeren SA, Vervoort J, van Berkel WJ. 1997. Purification and properties of 4-hydroxybenzoate 1-hydroxylase (decarboxylating), a novel flavin adenine dinucleotide-dependent monooxygenase from *Candida parapsilosis* CBS604. J Bacteriol 179:6680–6687. <https://doi.org/10.1128/jb.179.21.6680-6687.1997>.
 37. Holesova Z, Jakubkova M, Zavadiakova I, Zeman I, Tomaska L, Nosek J. 2011. Gentisate and 3-oxoadipate pathways in the yeast *Candida parapsilosis*: identification and functional analysis of the genes coding for 3-hydroxybenzoate 6-hydroxylase and 4-hydroxybenzoate 1-hydroxylase. Microbiology 157:2152–2163. <https://doi.org/10.1099/mic.0.048215-0>.
 38. Yang YL, Zhou H, Du G, Feng KN, Feng T, Fu XL, Liu JK, Zeng Y. 2016. A monooxygenase from *Boreostereum vibrans* catalyzes oxidative decarboxylation in a divergent vibralactone biosynthesis pathway. Angew Chem Int Ed Engl 55:5463–5466. <https://doi.org/10.1002/anie.201510928>.
 39. Koskineniemi H, Metsä-Ketelä M, Dobritzsch D, Kallio P, Korhonen H, Mäntsälä P, Schneider G, Niemi J. 2007. Crystal structures of two aromatic hydroxylases involved in the early tailoring steps of angucycline biosynthesis. J Mol Biol 372:633–648. <https://doi.org/10.1016/j.jmb.2007.06.087>.
 40. Wang P, Bashiri G, Gao X, Sawaya MR, Tang Y. 2013. Uncovering the enzymes that catalyze the final steps in oxytetracycline biosynthesis. J Am Chem Soc 135:7138–7141. <https://doi.org/10.1021/ja403516u>.
 41. Bosserman MA, Downey T, Noinaj N, Buchanan SK, Rohr J. 2013. Molecular insight into substrate recognition and catalysis of Baeyer-Villiger monooxygenase MtmOIV, the key frame-modifying enzyme in the biosynthesis of anticancer agent mithramycin. ACS Chem Biol 8:2466–2477. <https://doi.org/10.1021/cb400399b>.
 42. Liu LK, Abdelwahab H, Martin Del Campo JS, Mehra-Chaudhary R, Sobrado P, Tanner JJ. 2016. The structure of the antibiotic deactivating, N-hydroxylating rifampicin monooxygenase. J Biol Chem 291:21553–21562. <https://doi.org/10.1074/jbc.M116.745315>.
 43. Min J, Zhang JJ, Zhou NY. 2015. A two-component *para*-nitrophenol monooxygenase initiates a novel 2-chloro-4-nitrophenol catabolism pathway in *Rhodococcus imtechensis* RKJ300. Appl Environ Microbiol 82:714–723. <https://doi.org/10.1128/AEM.03042-15>.
 44. Sambrook J, Russell D. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
 45. Chen Q, Wang CH, Deng SK, Wu YD, Li Y, Yao L, Jiang JD, Yan X, He J, Li SP. 2014. Novel three-component Rieske non-heme iron oxygenase system catalyzing the N-dealkylation of chloroacetanilide herbicides in sphingomonads DC-6 and DC-2. Appl Environ Microbiol 80:5078–5085. <https://doi.org/10.1128/AEM.00659-14>.
 46. Petersen TN, Brunak S, von Heijne G, Nielsen H. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods 8:785–786. <https://doi.org/10.1038/nmeth.1701>.
 47. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729. <https://doi.org/10.1093/molbev/mst197>.
 48. Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425.
 49. Hall T. 2004. Bioedit version 6.0.7. Department of Microbiology, North Carolina State University, Raleigh, NC.
 50. Rost B. 1996. PHD: predicting one-dimensional protein structure by profile-based neural networks. Methods Enzymol 266:525–539. [https://doi.org/10.1016/S0076-6879\(96\)66033-9](https://doi.org/10.1016/S0076-6879(96)66033-9).
 51. Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. Nat Methods 9:357–359. <https://doi.org/10.1038/nmeth.1923>.
 52. Anders S, Pyl PT, Huber W. 2015. HTSeq—a Python framework to work with high-throughput sequencing data. Bioinformatics 31:166–169. <https://doi.org/10.1093/bioinformatics/btu638>.
 53. Young MD, Wakefield MJ, Smyth GK, Oshlack A. 2010. Gene ontology analysis for RNA-seq: accounting for selection bias. Genome Biol 11:R14. <https://doi.org/10.1186/gb-2010-11-2-r14>.
 54. Higuchi R. 1989. Using PCR to engineer DNA: principles and applications for DNA amplification, p 61–70. In Ehrlich H (ed), PCR technology. Palgrave Macmillan, Basingstoke, United Kingdom.
 55. Fontecave M, Eliasson R, Reichard P. 1987. NAD(P)H: flavin oxidoreductase of *Escherichia coli*. A ferric iron reductase participating in the generation of the free radical of ribonucleotide reductase. J Biol Chem 262:12325–12331.
 56. Bergmann JG, Sainik J. 1957. Determination of trace amounts of chlorine in naphtha. Anal Chem 29:241–243. <https://doi.org/10.1021/ac60122a018>.
 57. Louie TM, Webster CM, Xun L. 2002. Genetic and biochemical characterization of a 2,4,6-trichlorophenol degradation pathway in *Ralstonia eutropha* JMP134. J Bacteriol 184:3492–3500. <https://doi.org/10.1128/JB.184.13.3492-3500.2002>.
 58. Kolvenbach BA, Dobrowinski H, Fousek J, Vlcek C, Schäffer A, Gabriel FL, Kohler HP, Corvini PF. 2012. An unexpected gene cluster for downstream degradation of alkylphenols in *Sphingomonas* sp. strain TTNP3. Appl Microbiol Biotechnol 93:1315–1324. <https://doi.org/10.1007/s00253-011-3451-8>.
 59. Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM, Peterson KM. 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166:175–176. [https://doi.org/10.1016/0378-1119\(95\)00584-1](https://doi.org/10.1016/0378-1119(95)00584-1).
 60. Quandt J, Hynes MF. 1993. Versatile suicide vectors which allow direct selection for gene replacement in gram-negative bacteria. Gene 127:15–21. [https://doi.org/10.1016/0378-1119\(93\)90611-6](https://doi.org/10.1016/0378-1119(93)90611-6).
 61. Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L. 2010. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat Biotechnol 28:511–515. <https://doi.org/10.1038/nbt.1621>.
 62. Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254. [https://doi.org/10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3).